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Cloning of Haloacid Dehalogenase Gene from *Bacillus cereus* Local Strain with the Addition of Restriction Sites

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Abstract

Previous studies had successfully isolated and characterized the haloacid dehalogenase gene from *Bacillus cereus* local strain, namely *bcfd1* gene. In the further research, this gene would be sub cloned into expression vector in order to analyse its expression. However, this gene could not be directly sub cloned because it doesn't have suitable restriction sites that facilitate correct orientation of cloning. Therefore, the addition of suitable restriction sites at both end of the gene was necessary. The research is started by designing specific pair of primers to amplify the *bcfd1* gene from *Bacillus cereus* chromosome by adding *Eco*RI on forward primer and *Hind*III on reverse primer. The 870 bp of *bcfd1* gene code for haloacid dehalogenase with suitable restiction sites has been successfully cloned into the pGEM-T Easy cloning vector. The recombinant clone that was obtained from screened by ampicillin resistant and ß-galactosidase activity was confirmed by size screening, restriction analysis, and re-PCR. This clone already to be performed for the further sub cloning process in order to get the right cloning direction.

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Keywords: Bacillus cereus; cloning; haloacid dehalogenase; restriction sites.

Nomenclature

PCR	Polymerase chain reaction
MCA	Monochloro acetate
bcfd1	gene encoding haloacid dehalogenase from *Bacillus cereus* local strain
LB	Luria-Bertani medium

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1. Introduction

Organohalogen are organic compounds widely used as pesticides active substances, plasticizer, precursors in chemicals syntheses, and as organic solvents in many chemical industries. Organohalogen belong to one of xenobiotic compounds that caused environmental pollution and harmful to human health due to its toxicity, persistence, and its possible transformation to other toxic metabolites[1]. In addition, these compounds can spread over the biosphere, seep into the grounds, accumulated in the ground water, and may cause long-term pollution[2]. In long-term exposure to human, these compounds could caused kidneys dysfunction, liver and nervous systems damage, as well as decreasing the immunity and developing cancer. Therefore, organohalogen is being one of public concerns due to its adverse effects on life[3].

One effort to eliminate or reduce the organohalogen pollutants in the environment is bioremediation, utilizing microorganisms as agents to degrade the pollutants[4]. Microorganisms that produced dehalogenase could metabolized organohalogen and transformed to harmless and non-toxic metabolites[5]. Hence, these microorganisms are potentially to be used to overcome organohalogen pollutants[6]. There are many bacterial genuses with dehalogenase activity are already isolated, among other are *Moraxella sp.*[7], *Pseudomonas sp.*[8], *Bulchordia sp.*[9], *Rhizobium sp.*[1], *Bacillus sp.*[10], *Xanthobacter sp.*[11],and *Rhodobacteraceae*[6].

Haloacid dehalogenase is one of dehalogenase group that has been widely studied. Haloacid dehalogenase (*EC* 3.8.1) or halidohydrolyse is hydrolase class that breaks halogen-carbon bond on halogenated aliphatic acid[12]. Haloacid dehalogenase is divided into two major groups, namely DehI and DehII[13]. DehI is the L-haloacid dehalogenase which only works on the haloacid substrate with L enantiomer form which inversion occurs on C2 configuration and generates D-hydroxyalcanoat acid[14]. DehII consist of D-haloacid dehalogenase which only works on haloacid substrate with D enantiomer form whereas on the other hand D/L-haloacid dehalogenase can works on both enantiomer form haloacid substrate.

In wildtype bacterial strains, dehalogenase is generally expressed in low level, hence it is not efficient to reduce the organohalogen pollutants. The development of molecular recombinant DNA technology provides a solution of this problem. Production of several potential enzymes in large quantities have been succesfully performed by this technique, using an approach of identifying the gene encoding the desired enzyme and then expressing that gene in new expression system with higher expression ability.

Previous studies had succesfully isolated and characterized the haloacid dehalogenase gene from *Bacillus cereus* local strain obtained from Agriculture Research and Development Association of Indonesia. This gene had been cloned in pGEM-T Easy, and named as *bcfd1* gene[15,16]. This gene would be sub cloned into expression vector in order to analyse its expression. However, this gene could not be directly sub cloned because it doesn't have suitable restriction sites that facilitate correct orientation of cloning. The correct orientation of the gene in its expression vector plays an important role in expression. This research aims to clone this haloacid dehalogenase gene with addition of suitable restriction sites at both end of the gene, so that its could be further sub cloned into certain expression vector.

2. Materials and Methods

2.1. Bacterial strains, plasmid, and growth conditions

Bacillus cereus local strain was obtained from Agriculture Research and Development Association of Indonesia was used as source of chromosomal DNA. This bacterium was grown aerobicaly at 37 ^{o}C in an Luria-Bertani (LB) medium that contained 0.5% (w/v) yeast extract, 1% (w/v) trypton and supplemented with 5 mM monochloro acetate (MCA). *E. coli* TOP10 was used as a host strain for gene cloning. The recombinant clones were grown at 37 ^{o}C in LB medium supplementd with 100 µg/ml ampicillin. The pGEM-T Easy (Promega) was used as a cloning vector.

2.2. Enzymes and Chemicals

Restriction endonucleases (*EcoRI* and *Hind*III), T4 DNA ligase and DNA ladder were purchased from

Promega. Oligonucleotide primers were ordered from 1stBASE, Malaysia. The positive transformants were screened in LB solid medium supplemented with 20 µg/ml 5-bromo-4-chloro-3-indolyl-ß-D-galactoside (X-gal) was purchased from Promega. Monochloro acetate acid was purchased from Merck. Isopropyl-thio-ß-D-galactoside (IPTG) was used to induce of the gene expression under control of *lac* promoter control. All other chemicals were of analytical grade.

2.3. Primers design, gene amplification, and cloning

Primers were designed based on the nucleotide sequences information of *bcfd1* gene that code haloacid dehalogenase from *Bacillus cereus* local strain which was obtained from previous studies. Specific primers were designed with the addition of restriction enzyme sites at both end of the gene.

Amplification of *bcfd1* gene was performed on *Bacillus cereus* strain local chromosomal DNA as a template via *PCR,* using specific primers that have been designed. The amplification reaction was mixed in 20 µl by using *Kappa Taq Ready Mix PCR* Kit (Kappa Biosystem) which composition according to the company protocol. PCR reaction was initiated with predenaturation at 94 ^{o}C for 4 min, and this was followed by 34 cycles of denaturation at 94 ^{o}C for 15 s, annealing at 52 ^{o}C for 30 s, extention at 72 ^{o}C for 1 min. Reaction ended by final extention at 72 ^{o}C for 5 min. The PCR amplification product was confirmed by electrophoresis on a 1% agarose gel.

The PCR amplification product then was ligated into linear cloning vector, pGEM-T *Easy* using T4 DNA ligase. Ligation products then transformed into *E. coli* TOP10 competent using heat shock method (Sambrook and Russel, 2001). Competent cells of *E. coli* TOP10 were prepared by standard $CaCl_2$ procedure[17]. The *E. coli* TOP10 transformants were grown in LB solid medium supplemented with 100 µg/mL ampicillin, 0,1 mM IPTG and 20 µg/ml X-gal and positive transformants were selected based on blue-white screening. The positive recombinant clones were confirmed by size screening, restriction analysis, and re-PCR. Plasmid DNA was isolated by alkaline lysis method[17].

3. Results and Discussion

3.1. Specific Primers Design

In previous studies, *bcfd1* gene that had been cloned dose not have suitable restriction sites that facilitate correct orientation of its further sub cloning into expression vector. These restriction sites will help us to direct the *bcfd1* gene ligation into expression vector at the correct orientation. Gene direction in the expression vector will determine the success of its further expression. This gene will be sub cloned into expression vector pET-30a. Therefore, the restriction sites will be choosed must consider the existing restriction sites on the pET-30a and *bcfd1* gene. The restriction sites allowed to be used are the restriction sites that exist within multiple cloning site in the destination vector but does not exist within the cloned gene.

Restriction sites were added at the gene via PCR using appropriate primers. Restriction site was added on the forward primer is the restriction site located after the promoter, whereas on the reverse primer is the the restriction site located after forward primer and promoter. Forward primer contains a sequence initiation codon, while reverse primer contains a termination codon of gene, so that the initiation codon must be located exactly after promoter in order to express that gene. This method has been widely used in expressing recombinant genes including dehalogenase genes[1,6].

The primers characteristics can bee seen in Table 1. The restriction sites of *Eco*RI and *Hind*III were added at forward and reverse primer respectively. The restriction site added are underline. These enzymes are already common used among reasearcher, so it is not difficult to obtain.

Table 1. Specific primers design for amplification *bcfd1* gene

Primer	Nucleotide sequence (5'→3')	Length	Tm (˚C)	% GC
Forward	GCAGAATTCATGGATGGAACACTACTATC	29	57,2	41,4
Reverse	GCGAAGCTTTTATTTACTAGATGAAGTTTG	30	54,8	33,3

3.2. Gene amplification and cloning

Amplification product of *bcfd1* gene using specific primers via PCR yields of 870 bp. This result was seen as a single DNA band on the electrophoregram approximately at 870 pb as shown in Fig. 4 line 2. Ligation of DNA fragment with plasmid pGEM-T Easy could be directly perfomed without prior treatment due to it has complementary overhang ends. DNA fragment was yield via PCR using DNA *Taq* polymerase always have overhang A at 3' end, whereas plasmid pGEM-T *Easy* is a linear plasmid which have overhang T at 5'end. The recombinant plasmid harbored *bcfd1* gene was named pGEM-*bcfd1*.

3.3. Recombinant plasmid pGEM-bcfd1 confirmation

The *E. coli* TOP10 transformants were screened by ampicilin resistant and ß-galactosidase activity and positive recombinant clone was confirmed by size screening, restriction analysis, and re-PCR.

3.3.1 Blue-white screening

The *E. coli* TOP10 transformants were grown in selective medium can be identified by its color, cell with blue color and white color (Fig. 1). The transformants that carried recombinant plasmid pGEM-T *Easy* contains insert appear as white color, whereas the blue one are the transformants that carried plasmid pGEM-T Easy without insert.

The emergence of blue color in colonies due to the ß-galactosidase activity brought by the plamsid pGEM-T Easy. This enzyme breaks ß-glycosidic bond between galactose and indole group in X-gal. Indole group which is released spontaniously dimerizes yields blue color compounds[18]. Gene *bcfd1* insertion into pGEM-T located within the *lacZ* gene, encode ß-galactosidase, hence change its reading frame. As a result ß-galactosidase that produced becomes inactive and can not break X-gal, so that the cells appear as white colonies.

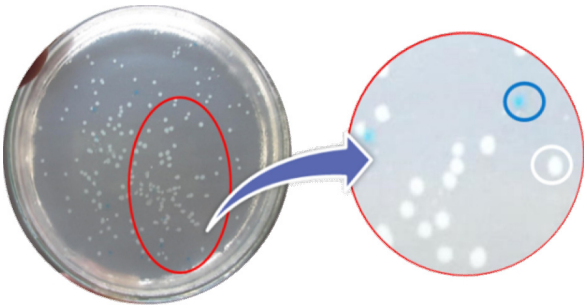

Fig. 1. Blue-white screening of *E. coli* TOP10 transformants

3.3.2. Size screening

Recombinant plasmids from several white and blue colonies were isolated and performed in the agarose gel electrophoresis to observe the migration distance of each plasmid. Size screening result of a number of recombinant plasmids can be seen in the Fig. 2. Plasmid DNA from blue colonies have a migration distance further than white one. Its means the size of the plasmid DNA from white colonies bigger than the plasmid from the blue one. Plasmid DNA from white colonies have big size due to the insertion and it was expected as recombinant plasmid contained *bcfd1* gene.

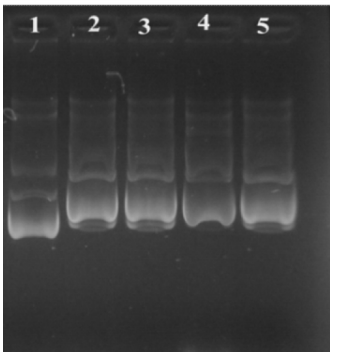

Fig. 2. *Size screening*: Electrophoregram of the plasmid DNA from trasnformant colonies. (*1 = plasmid from blue colonies, 2-5 = plasmid from white colonies 1,2,3,4*)

3.3.3. Restriction analysis

This analysis was performed to ensure that recombinant plasmids contained *bcfd1* gene. The positive recombinant plasmid was digested using two restriction enzymes, *EcoR*I and *Hind*III. This analysis also used to confirm whether the addition of restriction site at gene carried out succesfully or not. Plasmid DNA from white colonies number 1 was digested first with *Hind*III. Digestion products can be seen in the Fig.3. In the figure can be seen that there is only one plasmid DNA band appear after digest whith *Hind*III. Plasmid DNA band appear in the area around 4000 bp. Restriction analysis then continued by second digestion with *EcoR*I. Digestion products can be seen on Fig. 3 line 2.

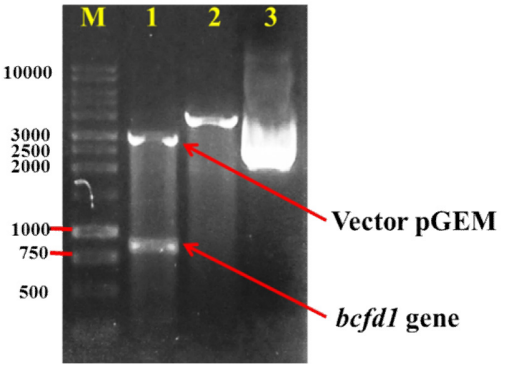

Fig. 3. Restriction analysis : Electrophoregram of digested plasmid DNA (*M = DNA Ladder 1 kb (Promega), 1 = digestion of pGEM-bcfd1 with HindIII and EcoRI, 2= digestion of pGEM-bcfd1 with HindIII, and 3= plasmid pGEM-bcfd1 without digestion*)

In the electrophoregram can be seen that there are two DNA bands, on the area approximately 3000 bp and 870 bp. DNA fragment on the area about 3000 bp is plasmid pGEM-T, whereas DNA fragment on the area about 870 bp is *bcfd1* gene. This analysis proved that the obtained recombinant plasmid contains a insert gene. It was estimated *bcfd1* gene.

3.3.4. Re-PCR of recombinant plasmid

Re-PCR was performed on positive recombinant plasmid as a template using same primers. Re-PCR product yields a single DNA band on a line with PCR product from *Bacillus cereus* local strain as a template (Fig. 4). The DNA fragment have the same size with *bcfd1* gene size, about 870 bp. It can be concluded that *bcfd1* gene has been succesfully cloned in to cloning vector pGEM-T Easy. The addition of restriction sites at both end of the gene also has been succesfully carried out.

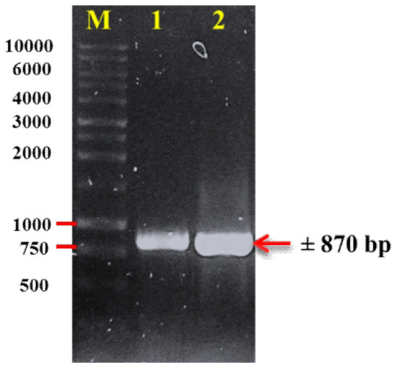

Fig. 4. *Re*-PCR: Electrophoregram of PCR products (*M = DNA Ladder 1 kb (Promega), 1 = amplicon of bcfd1gene using chromosomal DNA Bacillus cereus strain local as a template, 2= amplicon of bcfd1 gene using pGEM-bcfd1 as a template.*

4. Conclusion

The primers in order to add suitable restriction sites at *bcfd1* gene that code haloacid dehalogenase from *Bacillus cereus* local strain have been succesfully designed. The *bcfd1* gene contained suitable restriction sites has been succesfully cloned into cloning vector pGEM-T Easy. This clone already to be performed for the further sub cloning process in order to get the right cloning direction.

5. Acknowledgments

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